PERGAMON

Phytochemistry 50 (1999) 565–569

PHYTOCHEMISTRY

Free CoA as activator of 6-hydroxymellein synthase, a multifunctional polyketide biosynthetic enzyme in carrot

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Received 21 April 1998; revised 7 July 1998

Abstract

The activity of 6-hydroxymellein (6HM) synthase, a multifunctional polyketide biosynthetic enzyme in carrot, was appreciably inhibited in the presence of ATP: citrate lyase, a CoA-scavenging enzyme. Catalytic rate of the synthase markedly decreased when deuterium-labeled NADPH was employed in the enzyme reaction, however, the isotope effect was appreciably reduced by the addition of free CoA. These observations suggest that CoA is the activator of 6HM synthase both in low and high concentrations, and the compound functions as the acyl acceptor for proper entry of the substrates and enhances the ketoreducing reaction, the rate limiting step of 6HM biosynthesis. © 1998 Elsevier Science Ltd. All rights reserved.

Keywords: *Daucus carota*; Umbelliferae; Multifunctional polyketide biosynthetic enzyme; CoA; Deuterium isotope effect

6-Hydroxymellein (6HM) synthase is a multifunctional polyketide biosynthetic enzyme induced in carrot cells, and catalyzes the condensation of acetyl- and malonyl-CoAs (Kurosaki, Kizawa & Nishi, 1989). An NADPH-dependent ketoreduction of the carbonyl group takes place at the triketide intermediate stage to form the dihydroisocoumarin skeleton (Fig. 1a). We have recently demonstrated (Kurosaki, 1995) that the catalytically active form of the synthase is organized as the homodimer, and two multifunctional subunits are aligned in an antiparallel direction to form the two reaction centers. More recently, we have shown (Kurosaki, 1996) that a transacylase like domain is involved in the structure of 6HM synthase as a common primary binding site of the co-substrates, and both acetyl and malonyl moieties bind to OH group of Ser residue at this domain as O-esters (Fig. 1b). Then, the acyl groups are properly channeled to two SH groups, Cys-SH of condensation enzyme and cysteamine-SH of 4′-phosphopantetheine attached to acyl carrier protein (ACP), prior to the initiation of the condensation reaction (Kurosaki, 1996). It is assumed that 6HM synthase shares several common properties with multifunctional animal fatty acid synthase (FAS) (Kurosaki et al., 1989; Kurosaki, 1995, 1996). In this class of FAS, it is widely accepted (Wakil, 1989; Smith, 1994) that the loading of substrates is random, and both acetyl and malonyl moieties load onto Ser-OH at the transacylase structure at any stage of substrate entry and chain elongation. If an undesired acyl group attaches to this structure, it is rapidly exchanged to the proper one by the ester-exchanging reaction with CoA as the acyl acceptor. Therefore, low concentration of CoA functions as an activator of FAS (Smith, 1994). In contrast, it is also well known (Smith, 1994) that the activity of animal FAS is appreciably inhibited in the presence of relatively high concentrations of CoA. If CoA concentration rises too high, the equilibrium in the translocation of substrates between CoA- and enzyme-bound forms is shifted to CoA-esters to decrease the concentration of free CoA. Thus, FAS is starved of substrates and the catalytic reaction is impeded. In the present experiments, we attempted to examine the possible functional similarity of CoA in the catalytic reactions of 6HM synthase and FAS.

To examine the role of low concentrations of CoA generated during 6HM biosynthesis, this compound was quenched by coupling the reaction system with ATP: citrate lyase (Linn, Stark & Srere, 1980), and the

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PII: S0031-9422(98)00570-6

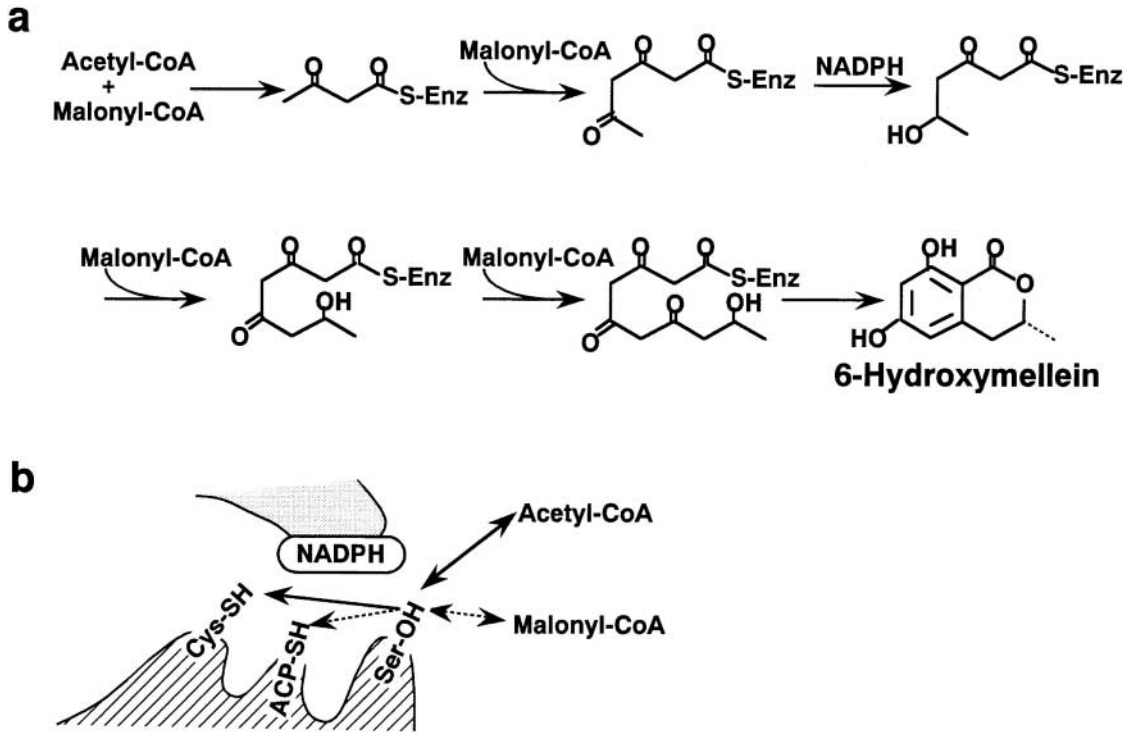

Fig. 1. (a) Catalytic reaction of 6HM synthase. 6HM synthase catalyzes the condensation of 1 acetyl-CoA and 4 malonyl-CoA, and the NADPH-dependent ketoreduction of the carbonyl group takes place at the triketomethylene intermediate stage. (b) Schematic presentation of the reaction center of 6HM synthase. The transacylase structure of 6HM synthase is the common primary binding site of the co-substrates, and both acetyl and malonyl moieties attach to Ser at this domain as o-esters. Then, acyl groups are properly channeled to Cys-SH and ACP-SH prior to the initiation of the condensation reaction.

effect of this CoA-scavenging enzyme on the synthase activity was examined (Table 1). The synthase activity was appreciably inhibited in the presence of catalytically active citrate lyase, the native enzyme plus its substrate: however, the lyase-induced inhibition was reversible and was restored to almost the control level by addition of 30 μM of free CoA. Recovery of the inhibition of the synthase activity was not solely observed for CoA, but pantetheine, another acyl acceptor, also showed an ability to reduce the citrate lyase-induced inhibition suggesting that the acyl acceptor is essential for the catalytic reaction of 6HM synthase. This observation agrees with our previous idea (Kurosaki, 1996) that the mechanism of substrate entry into 6HM synthase protein resembles that of multifunctional FAS. Indeed, since the transacylase-like domain of 6HM synthase is not able to recognize or distinguish acetyl and malonyl groups (Kurosaki, 1996), the rapid transfer back reaction of undesired acyl groups to appropriate acyl acceptors is essential for proper entry of the co-substrates.

As described above, the activity of animal FAS is appreciably inhibited when high concentrations of CoA are included in the reaction mixture (Smith,

Table 1
Effect of ATP: citrate lyase on 6HM synthase activity.

	Relative activity of 6HM synthase (%)[a]	
	Experiment 1	Experiment 2
Control	100	100
+ Citrate lyase	87	73
+ Boiled citrate lyase + citrate	88	99
+ Citrate lyase + citrate	34	21
+ Citrate lyase + citrate + CoA (30 μM)	82	93
+ Citrate lyase + citrate + pantetheine (30 μM)	88	65

[a] 6HM synthase activities of controls which received only Mg^{2+} and ATP as the additional factors were taken as 100%, and the results were expressed as percentages.

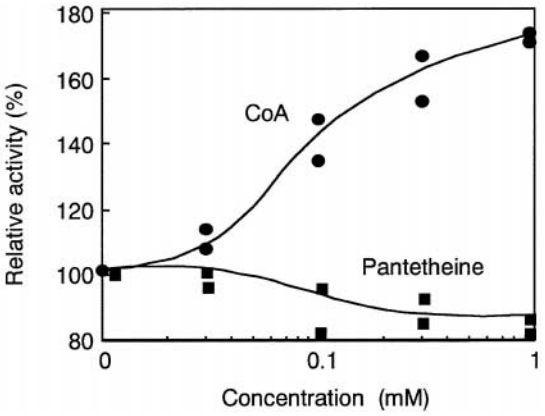

Fig. 2. Effect of high concentrations of acyl acceptors on 6HM synthase activity. 6HM synthase activity was determined in the presence of various concentrations of CoA or pantetheine, with results expressed as percentages relative to the control (100%) which did not receive acyl acceptors.

1994). However, this situation was found to be quite different in 6HM synthase, and, unlike in FAS, the activity of the synthase markedly increased in the presence of 0.1–1 mM of free CoA (Fig. 2). In contrast, another acyl acceptor, pantetheine, exhibited a slight inhibitory activity to the enzyme. These observations suggested the possibility that the CoA-induced activation of 6HM synthase is not due to the properties of the compound as an acyl acceptor. In order to elucidate the mechanisms responsible for this different mode of action of free CoA at high concentrations, we first determined the change in ratio of the synthase-bound forms of the co-substrates in the absence and presence of the acyl acceptors. Chemically modified 6HM synthase was prepared to simplify the assessment of the binding ability of the enzyme toward its substrates. Two SH groups at the reaction center of the synthase, Cys-SH and ACP-SH, were blocked with alkylating reagents according to the methods reported previously (Kurosaki, 1996). In this partially masked enzyme protein, it is expected that only the primary binding site of the acyl groups, Ser-OH of transacylase structure, remains free (Fig. 1b). As reported previously (Kurosaki, 1995, 1996), an attempt to estimate the chemical stoichiometry of the synthase-bound acyl groups versus the enzyme protein was unsuccessful because purity of the synthase in the samples varied in each batch of the enzyme preparations. In addition, a part of the synthase activity was lost during purification because of its instability (Kurosaki, 1995, 1996). Therefore, the experimental results were expressed as relative values in which the amount of acetyl group bound to the modified enzyme protein was taken as 1 in each set of the experiments. As shown in Table 2, addition of 300 μM of CoA resulted in the slight decrease in the enzyme-bound forms of acetyl and malonyl groups when these two substrates were added either independently or as 1:1 mixture (*ca* 70–90% of the controls). Addition of pantetheine also showed similar results and the slight decrease in the enzyme-bound forms of the substrates was observed. In animal FAS, it was reported (Stern, Sedgwick & Smith, 1982) that free CoA showed a potent inhibitory activity against the binding ability of the enzyme, and, by the addition of the compound, the ratio of the enzyme-loaded acyl groups decreased to the level of about 10% of controls. These different abilities of the two synthases in maintaining the substrates within the reaction pockets in the presence of the acyl acceptors might be due to the difference in the microstructures around the substrate entry sites.

As shown above, the equilibrium in the translocation of the substrates in 6HM synthase was shifted from the synthase-loaded to CoA-bound form by addition of free CoA. This observation implies that, at least at the substrate entry stage, CoA at high concentrations should function, if only weakly, as an inhibitor of the 6HM synthetic reaction as for FAS (Smith, 1994). Therefore, the possibility was suggested that CoA plays a role in a certain process other than the

Table 2
Effect of acyl acceptors on binding ability of 6HM synthase to its co-substrates

		Binding ability toward acyl-CoAs[a]					
		Experiment 1			Experiment 2		
		Control	+ CoA	+ Pantetheine	Control	+ CoA	+ Pantetheine
Acetyl-CoA		1[b]	0.73	0.80	1	0.82	0.85
Malonyl-CoA		0.90	0.81	0.82	1.27	0.87	0.81
Acetyl-CoA	Acetyl	0.56	0.40	0.41	0.44	0.36	0.40
+ malonyl-CoA	Malonyl	0.47	0.42	0.40	0.52	0.39	0.42

[a] Two SH groups at the reaction center of 6HM synthase were blocked by alkylation, and binding ability of the modified enzyme against acyl-CoAs was examined in the presence of 300 μM of CoA or pantetheine. Controls did not receive acyl acceptors.

[b] Results were expressed as relative values in which the amounts of acetyl group bound to the modified enzyme were taken as 1 in each set of the experiments.

Table 3
Effect of acyl acceptors on deuterium isotope effect in 6HM synthase reaction.

		Relative rate[a] (kH/kD)
[D]NADPH		5.2 ± 0.6[b]
[D]NADPH + CoA	(300 mM)	2.8 ± 1.0
	(30 mM)	4.3 ± 0.7
[D]NADPH + pantetheine	(300 mM)	5.1 ± 1.4
	(30 mM)	5.4 ± 0.9

[a] The results were presented as the means and s.d. obtained from three independent experiments.
[b] Previously reported values (Kurosaki et al., 1993) were presented as kH/kD for the reaction without acyl acceptors.

substrate entry in 6HM biosynthesis to enhance the synthase activity. In our previous work, it was demonstrated (Kurosaki, Itoh, Kizawa & Nishi, 1993) that the NADPH-dependent ketoreduction at the triketide intermediate stage (Fig. 1a) is the rate limiting step which determines the overall rate of the biosynthesis of the compound, and a marked deuterium isotope effect (kH/kD 5.2) was observed when the 4-pro-S-hydrogen of NADPH was replaced by deuterium. It might be expected that if CoA affects this partial reaction of 6HM synthase, even a low effect of the compound would significantly alter the apparent enzyme activity. Therefore, in the next experiments, we tested the effect of CoA on the catalytic rate of 6HM biosynthesis employing stereospecifically deuterium-labeled NADPH. The assay of the synthase was carried out in the presence of 1 mM of NADPH or 4-S-[4-D]NADPH, and the effect of CoA and pantetheine on the synthetic rate of 6HM was examined. As shown in Table 3, [D]NADPH-induced isotope effect was markedly reduced in the presence of 300 μM of free CoA, and kH/kD was estimated to be 2.8. The decrease in the isotope effect was also observed with 30 μM of CoA though the degree of the recovery from the reduced reaction rate was rather low (kH/kD, 4.3). Therefore, it is very likely that the enhancement of 6HM biosynthesis with high concentrations of CoA is, at least partly, caused by contribution of the compound to the rate limiting reaction. In contrast, addition of pantetheine did not affect the rate of 6HM synthesis. It is widely accepted (Richards, 1970) that when the C–H bond is replaced by the C–D bond, generally, the energy level of the ground state but not the transition state of the enzymatic reaction is lowered, and thus the energy barrier of the reaction increases. This fact strongly suggests that, in ketoreducing process of 6HM synthase, the energy level of the ground state of the synthase-NADPH complex elevates in the presence of high concentrations of CoA not only in C–D but also in C–H bond system. These observations, together with the results shown in Table 2, imply that CoA at high concentrations functions as both an inhibitor (for the substrate entry) and an activator (for the rate limiting reaction) in 6HM biosynthesis. The apparent property of high concentrations of CoA as the activator (Fig. 2) would result from the balance of these two opposite effects. The mechanism of the change in energy level of the ketoreducing reaction in the presence of CoA is not clear at present. In animal FAS, it was demonstrated (Strom, Galeos, Davidson & Kumar, 1979) that the activity of enoyl reductase markedly increases in the presence of 10 μM of free CoA, and several lines of evidence suggest that this phenomenon is caused by an allosteric effect of CoA molecule. However, the effect of CoA on ketoreductase activity in FAS was not tested in this experiment (Strom et al., 1979). Therefore, at present, it is uncertain whether or not CoA-induced enhancement of these two NADPH-dependent processes, enoyl reduction in FAS and ketoreduction in 6HM synthase, correlate with each other.

In conclusion, free CoA functions as an activator in the catalytic reaction of a multifunctional polyketide biosynthetic enzyme 6HM synthase both at low and high concentrations. It appears that the low concentration of CoA is essential as an acyl acceptor for proper entry of the co-substrates as is in fatty acid synthase. In the presence of high concentration of CoA, it is likely that the microstructure around the NADPH-associated ketoreducing domain of the synthase is altered, and the energy barrier of the rate limiting reaction of 6HM biosynthesis decreases. Further elucidation of the mechanisms of free CoA-induced activation of 6HM synthase is in progress in our laboratory.

1. Experimental

1.1. Chemicals

6HM was prepared by demethylating 6-methoxymellein, which was isolated from fungal-infected carrot, with BBr_3 in dry CH_2Cl_2 as reported previously in detail (Kurosaki et al., 1989). Chloroacetyl-CoA was synthesized according to the method of Kawaguchi, Yoshimura and Okuda (1981). 2-Chloroethylphosphonic acid, acetyl-CoA, malonyl-CoA, free CoA, pantetheine, NADPH, NADP and bovine serum albumin were purchased from Sigma while iodoacetoamide and glucose-1-D (98%) was obtained from Wako and Aldrich, respectively. Glucose-6-phosphate dehydrogenase and hexokinase were from Oriental Yeast. [2-^{14}C]Acetyl-CoA (sp. act. 2.1 GBq/mmol) and [2-^{14}C]malonyl-CoA (sp. act. 2.2 GBq/mmol) were from New England Nuclear. All other chemicals were reagent grade.

1.2. Induction, purification and assay of 6HM synthase

6HM synthase was induced in carrot root tissues by treatment with 2-chloroethylphosphonic acid, and the synthase was highly purified according to the methods described previously (Kurosaki, 1995, 1996; Kurosaki et al., 1993). Purity of the enzyme preparation was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and the results were reported previously (Kurosaki, 1995, 1996; Kurosaki et al., 1993). Protein concentrations were determined by the method of Bradford (1976). The standard assay mixture of 6HM synthase activity consisted of 10 mM K-Pi (pH 7.5), *ca* 4 pkat of the synthase, 0.1 mM acetyl-CoA, 0.1 mM [^{14}C]malonyl-CoA (3.7 kBq), 1 mM NADPH and 5 mM dithiothreitol (DTT) in 200 μl volume. In some experiments, the reaction was coupled with a CoA-scavenging system, and 1 mM ATP, 2 mM $MgCl_2$, 2 mM Na-citrate and *ca* 0.1 nkat of ATP: citrate lyase, which was partially purified according to the method of Stern et al. (1982), were added in a total volume of 260 μl. If necessary, 30 μM of CoA or pantetheine was also added into the assay mixture. The reaction was run at 37° for 30 min, and the radioactivities incorporated into 6HM were determined after the products were separated by TLC on a silica gel plate (Kurosaki et al., 1989; Kurosaki, 1995).

1.3. Modification and binding assay of 6HM synthase

Two SH groups at the reaction center of 6HM synthase were blocked by alkylation according to the method described previously in detail (Kurosaki, 1996). In brief, DTT was removed from the synthase preparation by dialysis, and Cys- and ACP-SHs were alkylated by incubation of the protein with 5 mM of iodoacetoamide plus 1 mM of chloroacetyl-CoA at 37° for 15 min. After alkylation, 7 mM DTT was added to the mixture to quench the excess SH inhibitors, and the sample was dialyzed against 20 mM K-Pi buffer containing 5 mM DTT (pH 7.0) to remove these reagents. Binding abilities of the modified 6HM synthase against its substrates were determined by incubation with [^{14}C]-labeled acyl-CoAs essentially according to the method described previously (Kurosaki, 1996). The assay mixture consisted of 10 mM K-Pi (pH 7.5), 10 μM of [^{14}C]acetyl-CoA or [^{14}C]malonyl-CoA (7.4 kBq), 1 mM of NADPH, 5 μg protein of the enzyme preparation (*ca* 50 pkat/assay) and 5 mM DTT in a total volume of 100 μl. If necessary, [^{14}C]acyl-CoAs was incubated in the presence of 10 μM of unlabeled acyl-CoAs. The reaction was run for 2 min at 37°, and was terminated by the addition of 500 μl of 2 M TCA. The protein was recovered by precipitation after the addition of 100 μg of bovine serum albumin as a carrier, with the resultant pellets denatured and subjected to SDS-PAGE (8% gels). After electrophoresis, the protein band corresponding to the 6HM synthase subunit (128 kDa) was identified with standard proteins, and gel slices containing the enzyme were excised with a razor blade (Kurosaki, 1996). These were subsequently immersed in 0.5 ml of solvable (New England Nuclear), and radioactivities determined.

1.4. Preparation of stereospecifically deuterium-labeled NADPH

4-S-[4-D]NADPH was prepared essentially by the method described by Wilken, King & Dyar (1975). NADP was reduced with glucose-6-phosphate dehydrogenase in the presence of [1-D]glucose-6-phosphate which was generated from glucose-1-D by the action of hexokinase. The labeled NADPH was purified by an ion exchange chromatography on a DEAE-Sephacel column (Pharmacia, 1.6×11 cm) with a linear gradient of NaCl (0–0.5 M) in a total 100 ml of 20 mM K-Pi buffer (pH 7.0).

References

Bradford, M. M. (1976). *Analytical Biochemistry, 72,* 248.

Kawaguchi, A., Yoshimura, T., & Okuda, S. (1981). *Journal of Biochemistry, 89,* 337.

Kurosaki, F. (1995). *Archives of Biochemistry and Biophysics, 321,* 239.

Kurosaki, F. (1996). *FEBS Letters, 379,* 97.

Kurosaki, F., Itoh, M., Kizawa, Y., & Nishi, A. (1993). *Archives of Biochemistry and Biophysics, 300,* 157.

Kurosaki, F., Kizawa, Y., & Nishi, A. (1989). *European Journal of Biochemistry, 185,* 85.

Linn, T. C., Stark, M. J., & Srere, P. A. (1980). *Journal of Biological Chemistry, 255,* 1388.

Richards, J. H. (1970). In: P. D. Boyer, *The enzymes, vol. II* (p. 321). New York: Academic Press.

Smith, S. (1994). *FASEB Journal, 8,* 1248.

Stern, A., Sedgwick, B., & Smith, S. (1982). *Journal of Biological Chemistry, 257,* 799.

Strom, K. A., Galeos, W. L., Davidson, L. A., & Kumar, S. (1979). *Journal of Biological Chemistry, 254,* 8153.

Wakil, S. J. (1989). *Biochemistry, 28,* 4523.

Wilken, D. R., King, H. L., Jr, & Dyar, R. E. (1975). *Journal of Biological Chemistry, 250,* 2311.